



Metabolic Engineering

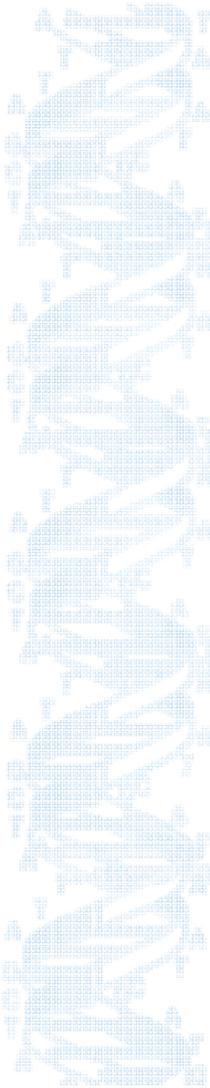
Metabolic Engineering of Yeasts for Ethanol Production from Biorefinery Hydrolysates

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Metabolic engineering is essential for the commercial fermentation of xylose

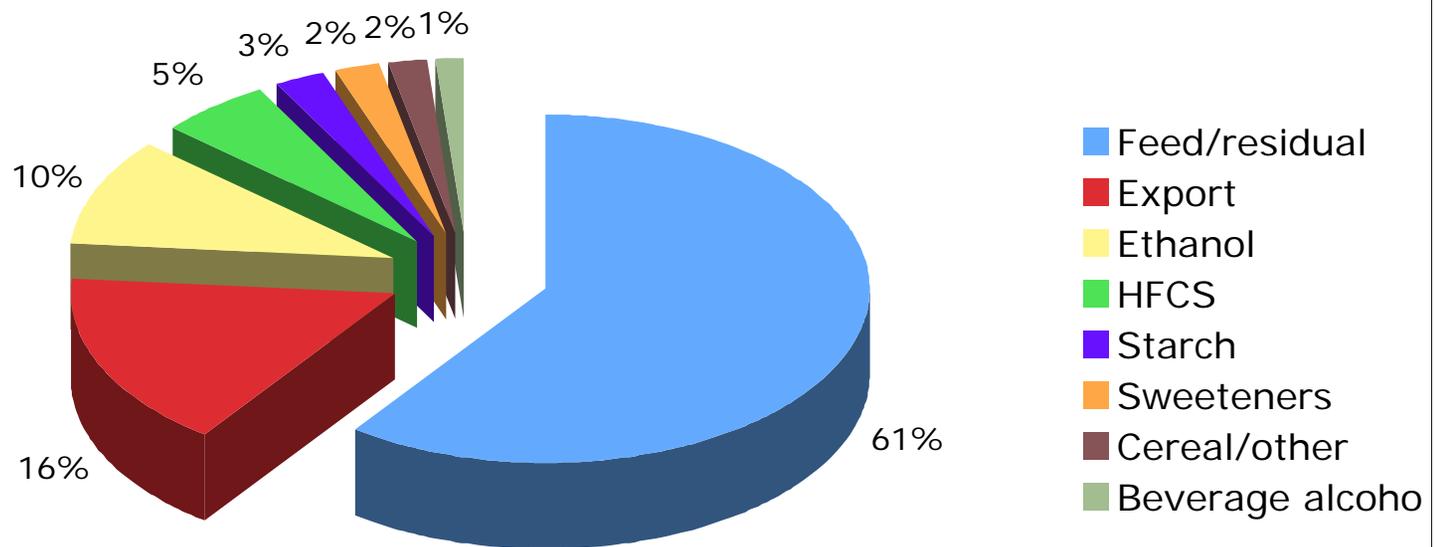
- Xylose is the second most abundant carbohydrate in nature
- It is readily recovered from lignocellulosic residues
- It is not fermented to ethanol with commercial rates and yields



Ethanol production from corn is growing rapidly

- The US ethanol industry produced about 3.4 billion gallons of ethanol in 2004
- This was a 21% increase over the 2.81 billion gallons in 2003...
- which in turn was 31% higher than production in 2002

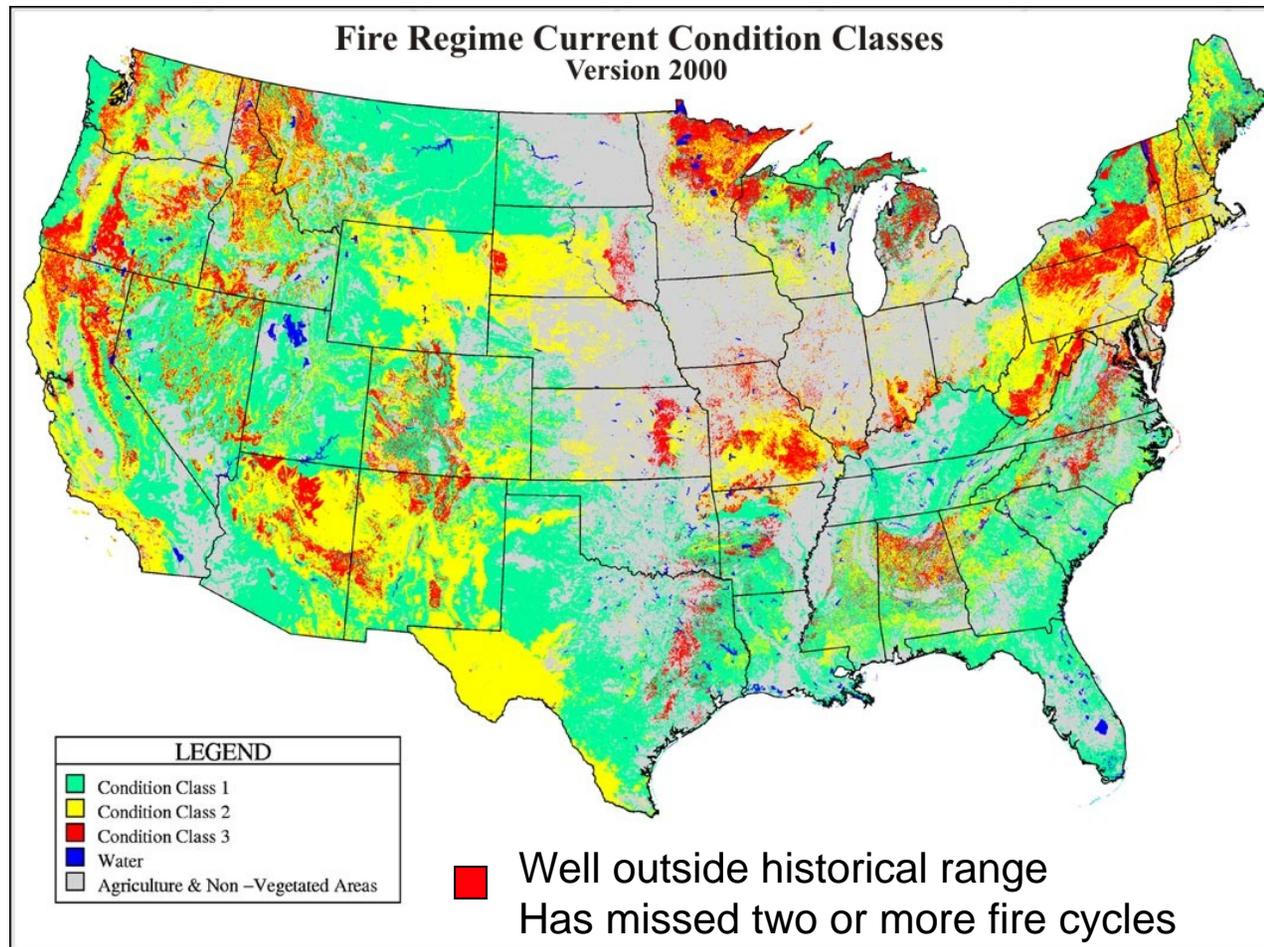
Only 10% of corn grain goes into fuel ethanol production



Each ton of grain results in 1 ton of residue, which is about 35% by weight xylose

Source: [USDA and industry](#)

Additional biomass could be obtained from wood



Adding value

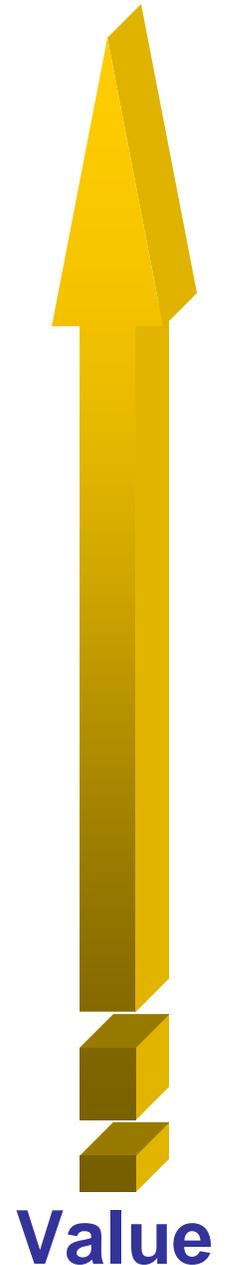
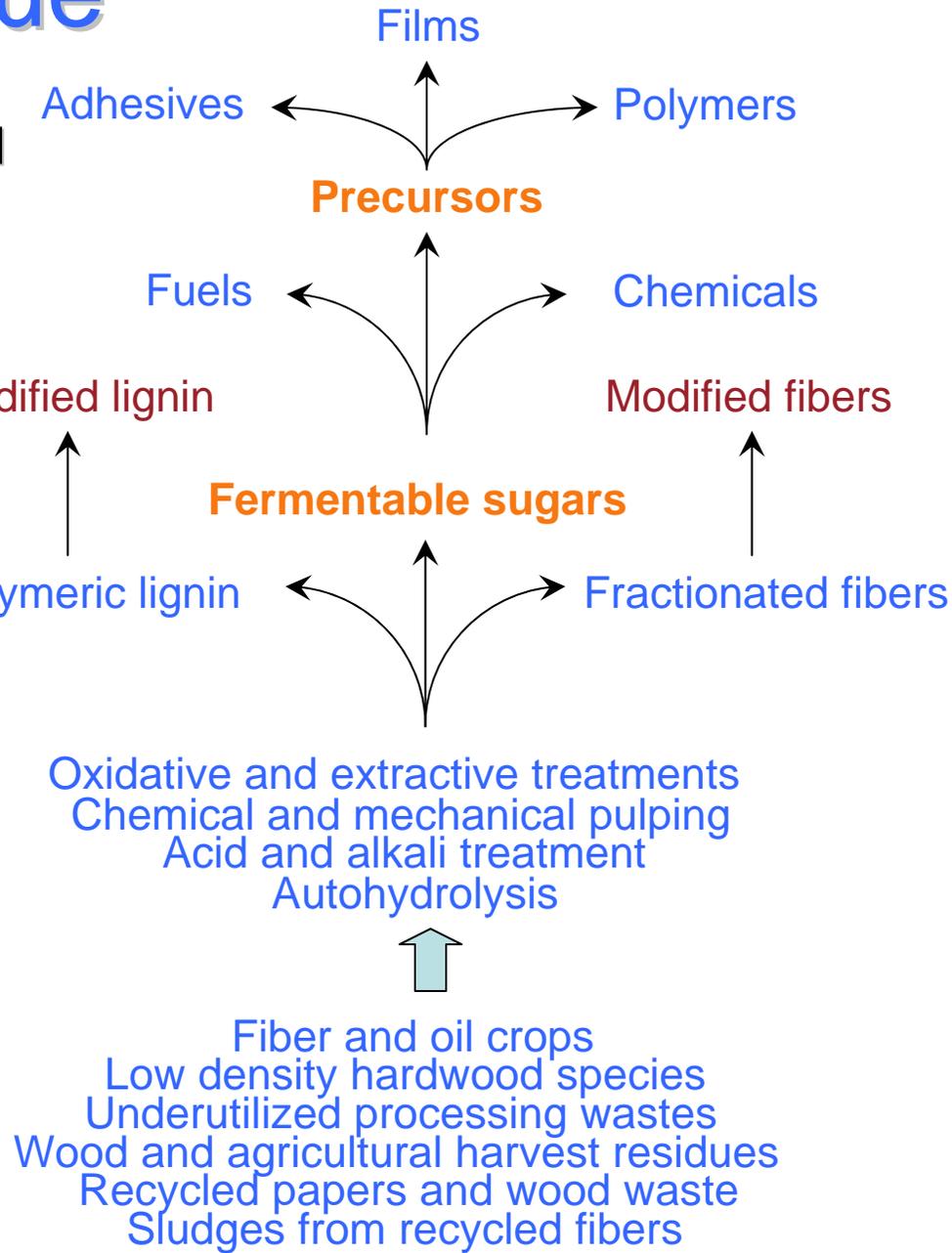


Biochemical and
Metabolic
Engineering

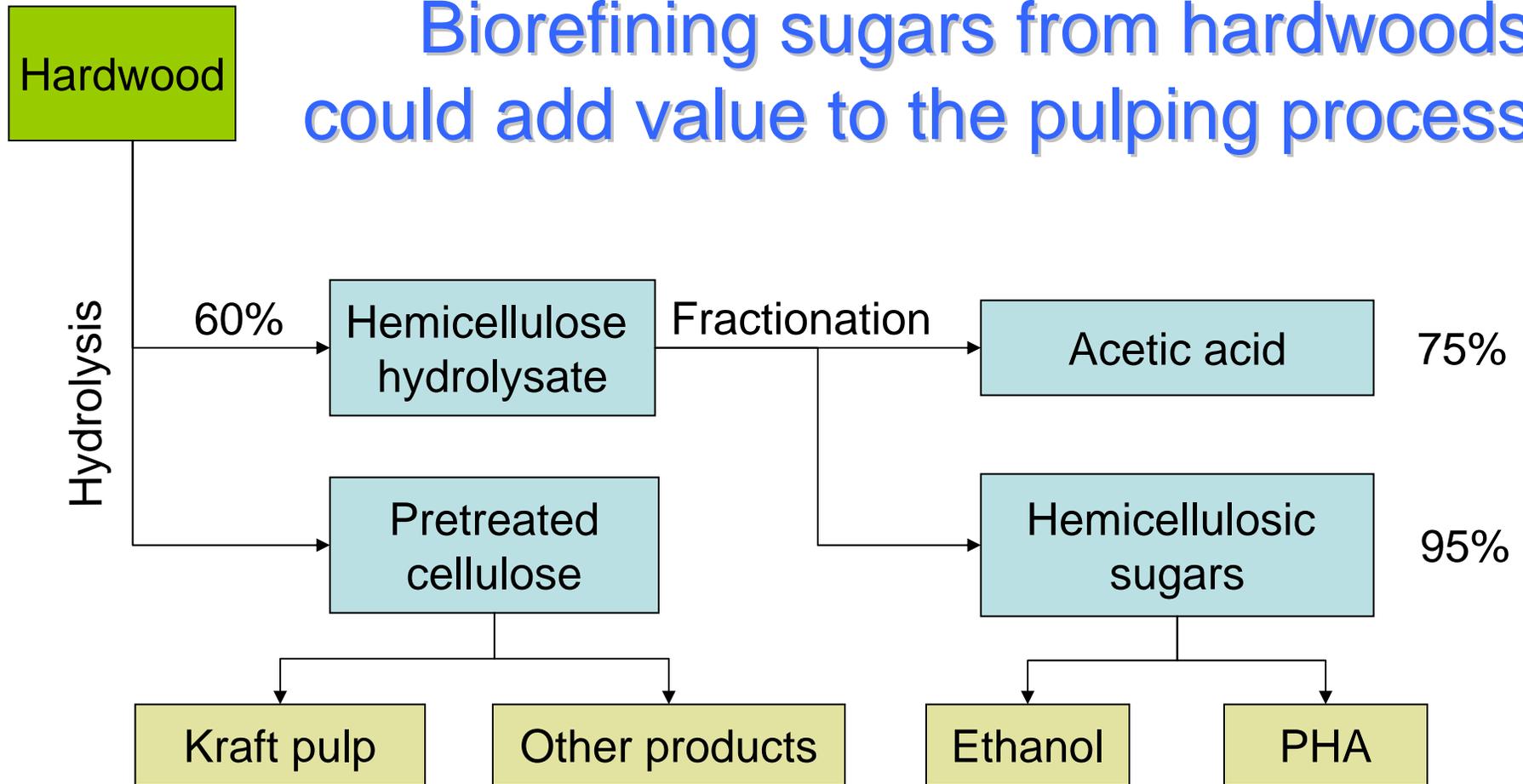
Enzymatic
Conversion

Pulping and
Pretreatments

Lignocellulosic
Feedstocks



Biorefining sugars from hardwoods could add value to the pulping process

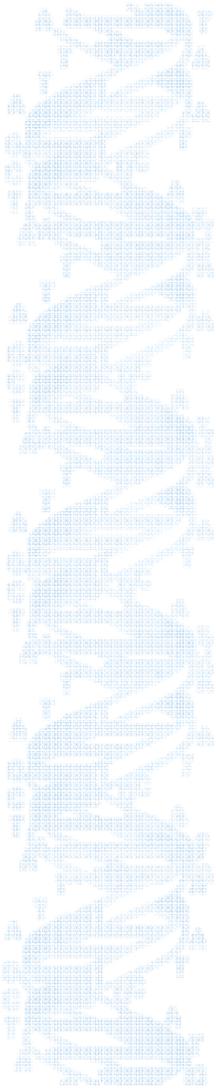


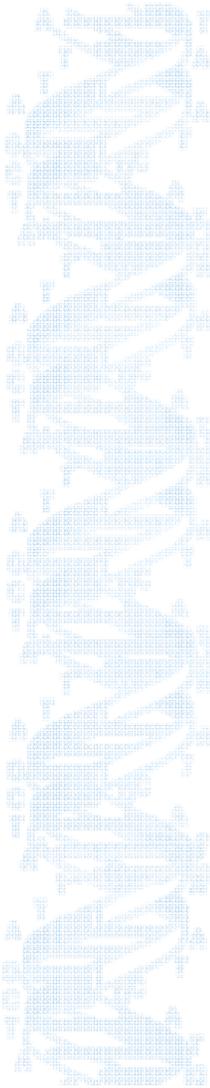
- Little heating value from HC
- Lower chemical use

- Higher loading on digester
- Generates new product streams

Where is our metabolic engineering work going?

- Analyzing genomic content and expression
 - *Pichia stipitis* genome complete
- Engineering whole pathways
 - Multiple (3 to 7) gene expression
- Optimizing metabolic flux
 - Multiple promoter optimization
- Understanding regulatory networks
 - Conducting genechip experiments



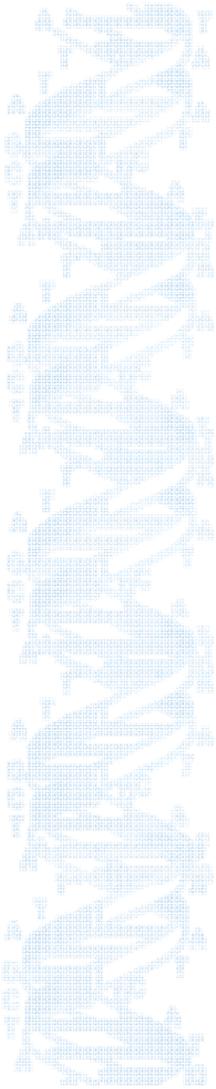


FPL research has developed improved yeasts for fermentation

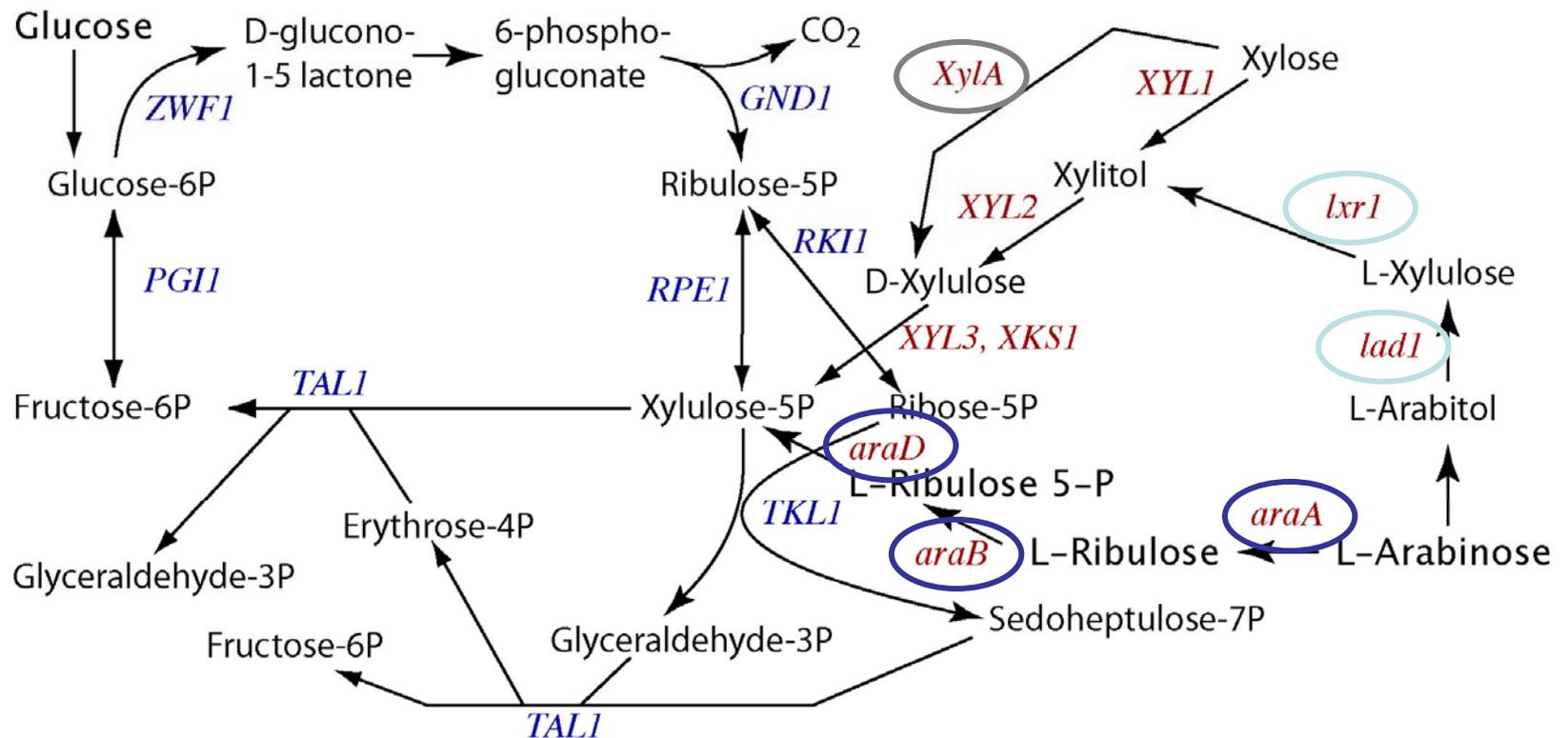
- Ethanol production increases if respiration is reduced
- Patents on engineered *P. stipitis* and *S. cerevisiae* have been filed
- Working with logen for commercial implementation

Major findings

- Transcript expression level affects the accumulation of intermediates
- Over expression of some enzymes can be toxic
- Effect of over expression depends on the genetic background
- Multiple genes contribute to optimal growth and fermentation



Xylose and arabinose metabolism engineered in *Saccharomyces*



Richard et al. 2003 FEMS Yeast Res. 2:185
 Becker & Boles 2003 AEM 69:4144
 Kuyper et al. 2004 FEMS Yeast Res. 6:655

Over-expression of *XYL3* inhibits growth on xylose

Expression vector Host strain *S. cerevisiae* 679 (Trp⁻)



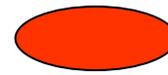
GAPDH promoter



S. cerevisiae 2 μ circle



GAPDH terminator



S. cerevisiae CEN/ARS

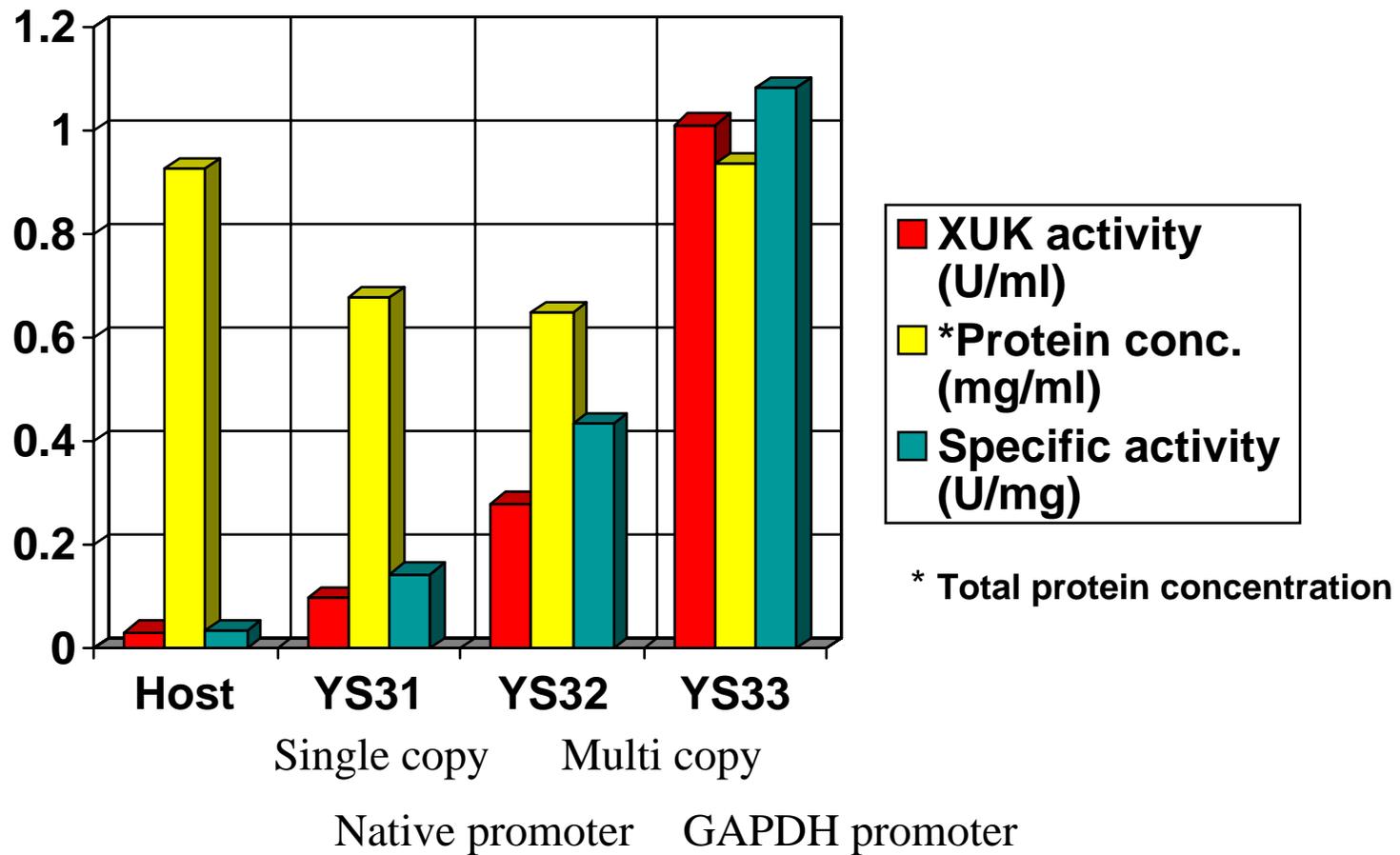


Native promoter

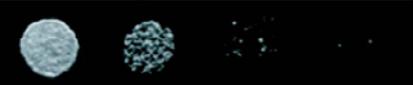
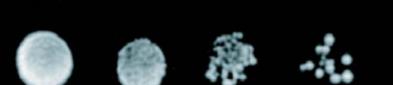
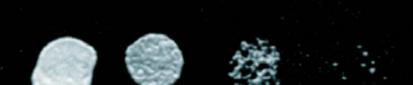


Native terminator

D-xylulokinase activity increases with promoter and copy number

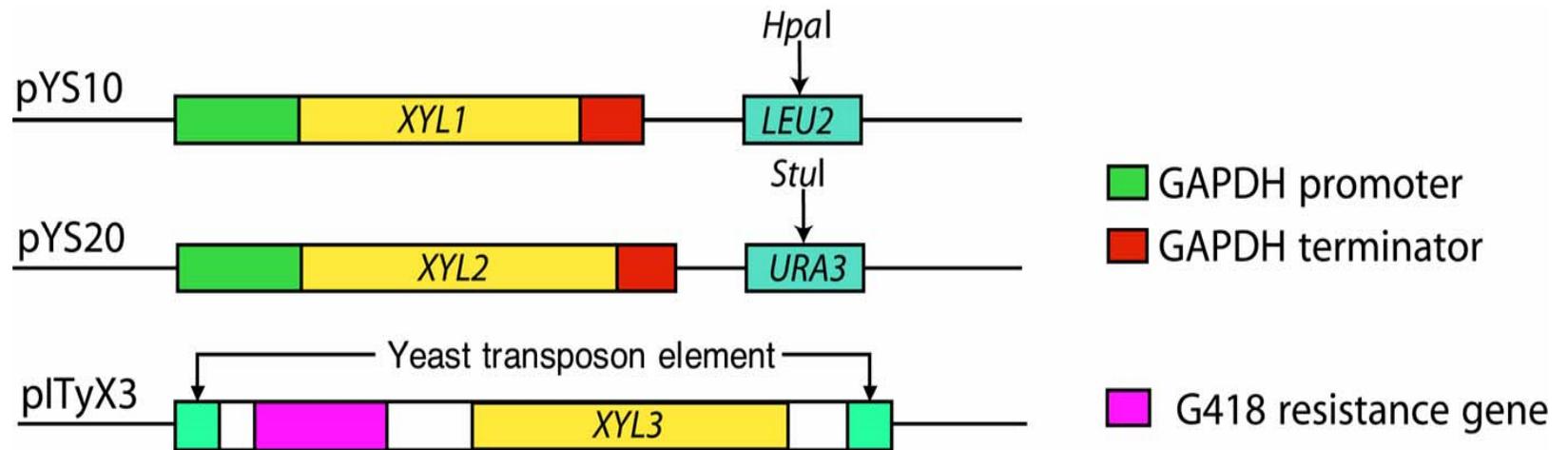


Xylulokinase over expression is toxic to cells during xylose metabolism

Constructs	Gene	Copy / Promoter	YSC-Glucose	YSC-Xylose	Xylulokinase activity (U/mg)	
					Glucose	Xylose
pYS31N	<i>PsXYL3</i>	S / N			0.18	0.19
pYS32N	<i>PsXYL3</i>	M / N			1.50	0.50
pYS32	<i>PsXYL3</i>	M / G			29.80	9.46
pYS42	<i>ScXKS1</i>	M / G			7.21	1.26
pRS314	Control	S			0.06	0.09
pRS424	Control	S			0.07	0.17
pYPR2831	Control	M / G			N/A	N/A

3 days 5 days

Integrating copies of *XYL3* gave optimal low expression

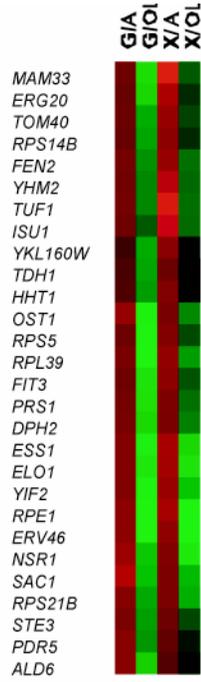


Three copies of *XYL3* were introduced

Four growth conditions were used for expression analysis

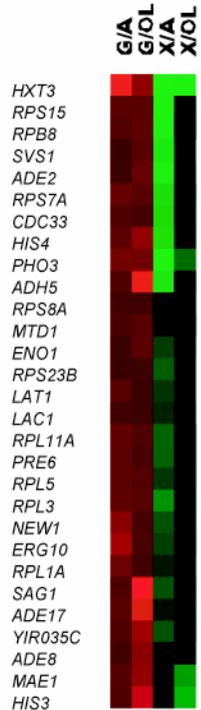
Conditions	High aeration	Low aeration
Glucose	2% Glucose (G/A)	4% Glucose (G/OL)
Xylose	2% Xylose (X/A)	4% Xylose (X/OL)

Cluster A:
Genes induced
under aerobic
conditions

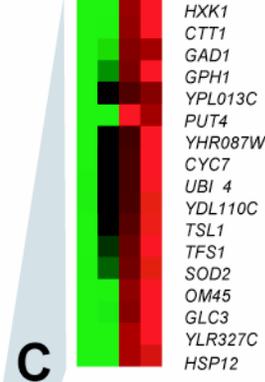
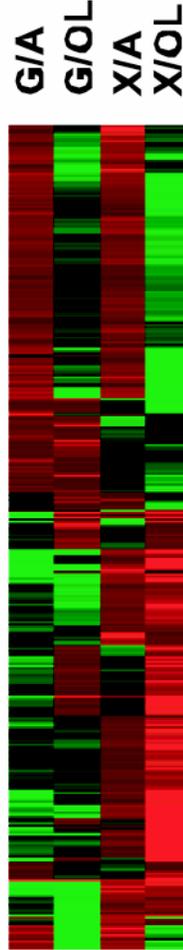


A

Cluster B:
Genes induced
by glucose



B



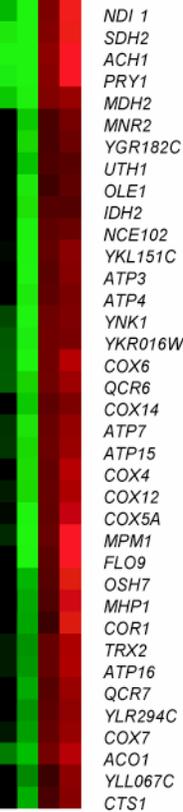
C

Cluster C: Genes
induced by
xylose



E

G/A
G/O/L
X/A
X/O/L

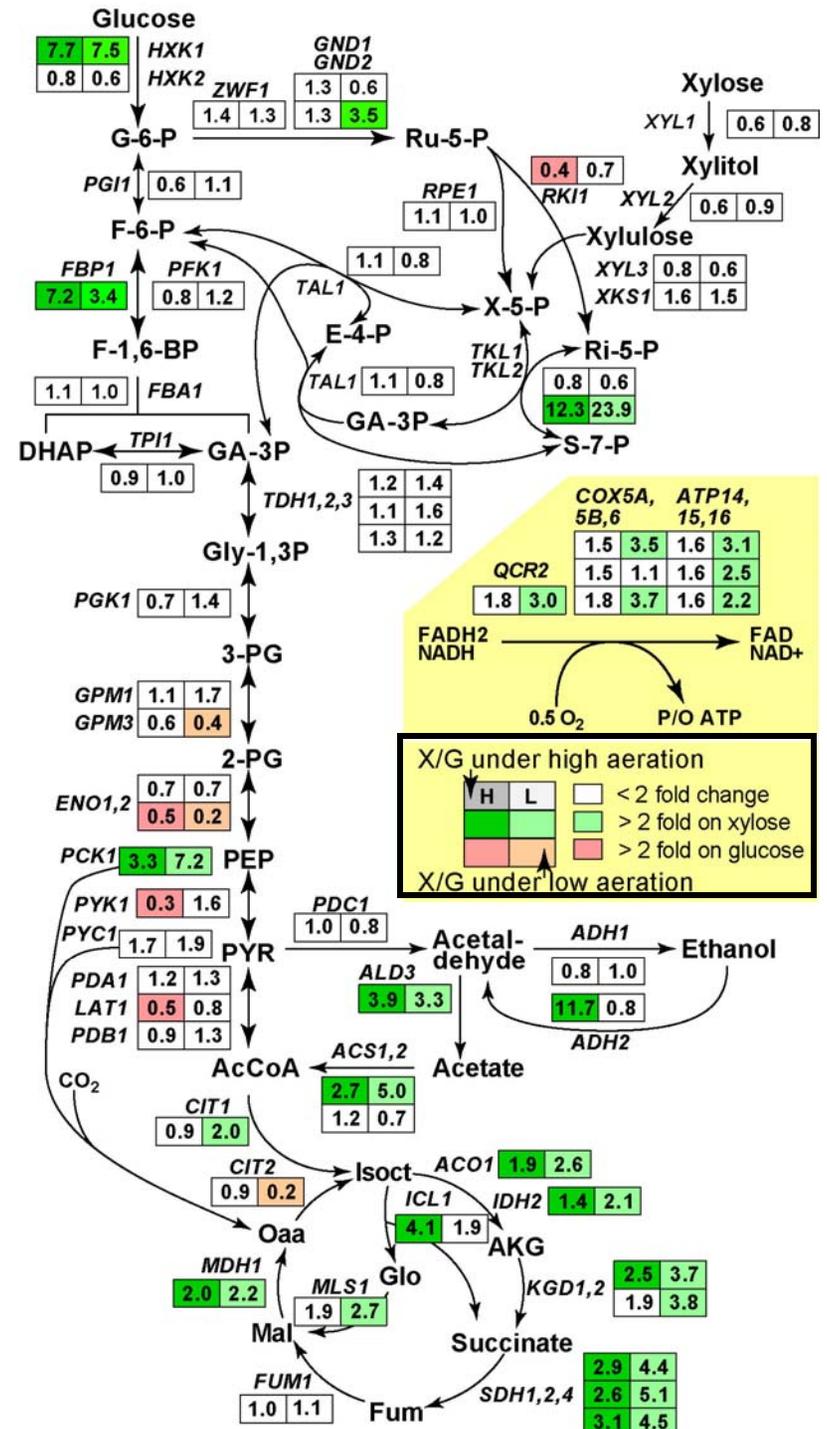


Cluster C: Genes
induced by
xylose

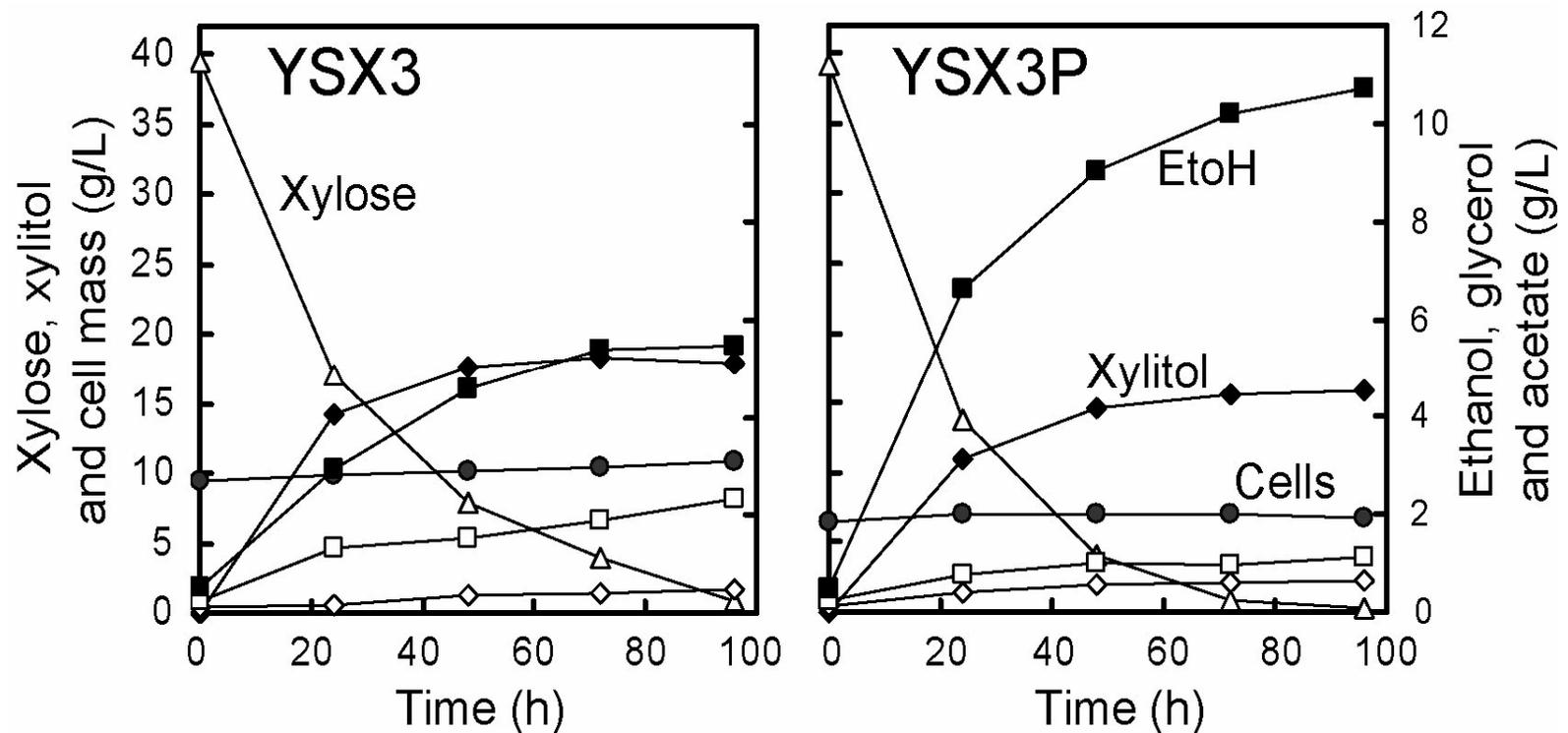
Cluster C: Genes
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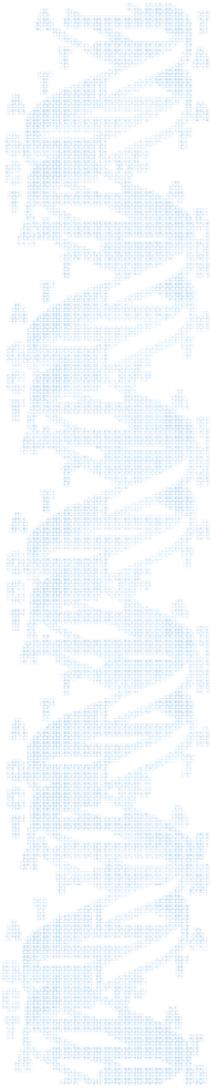
Transcript levels changed with carbon source and aeration

- Expressed as a ratio of abundance on xylose divided by abundance on glucose (X/G)
- Changes of less than 2-fold were not considered significant (white boxes)
- Red means higher on glucose; green means higher on xylose
- Dark (left) is under high aeration; light (right) is under low aeration



Ethanol production increases in a petite mutant of YSX3

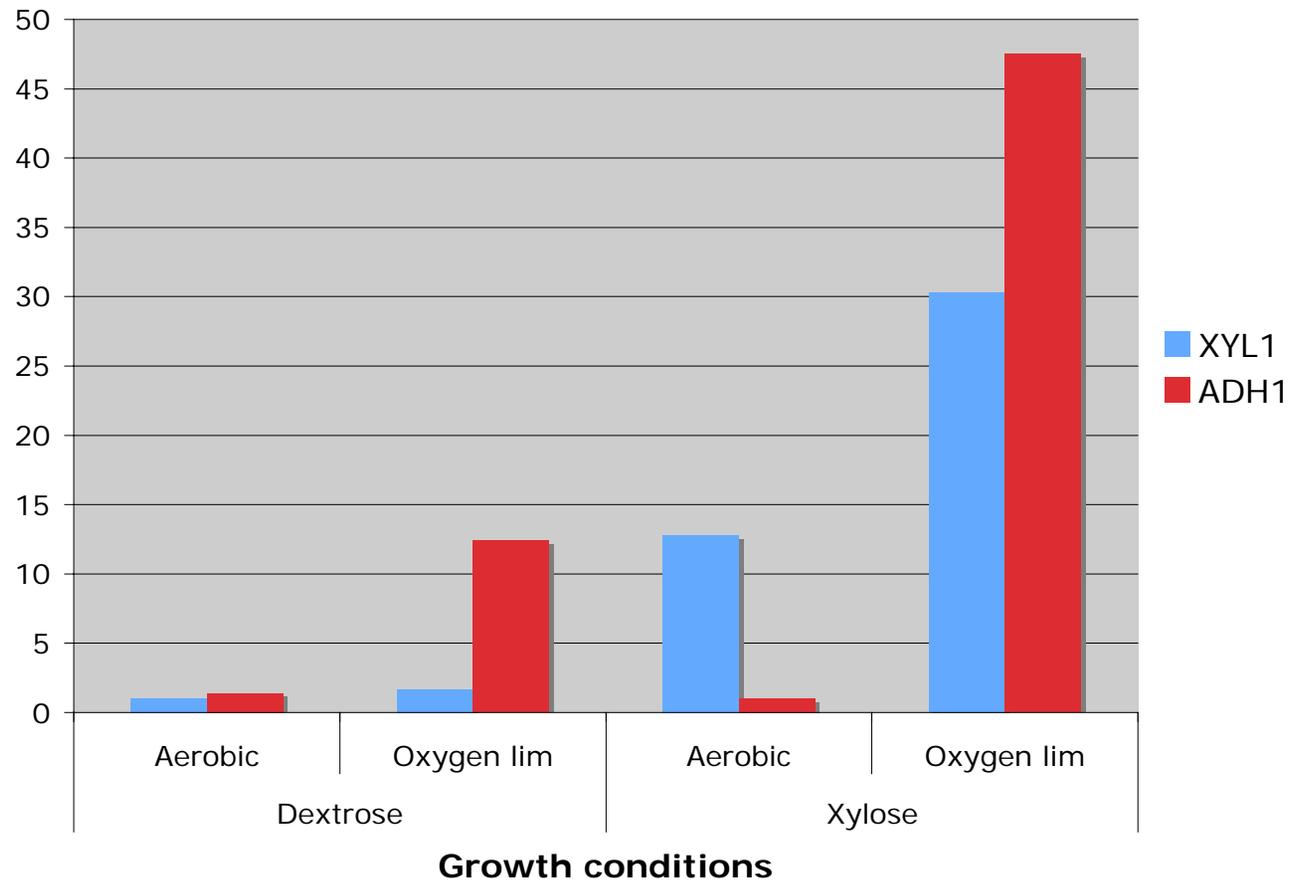




Shi21 (*cyc1*- Δ) shows higher ethanol yield and specific fermentation rates

Fermentation parameters	CBS 6054 (wild type)	FPL-UC7 (<i>ura3</i>)	FPL-Shi21 (<i>cyc1</i> - Δ)
Biomass yield ($\text{g}\cdot\text{g}^{-1}$)	0.16	0.17	0.09
Ethanol yield ($\text{g}\cdot\text{g}^{-1}$)	0.41	0.38	0.46
Specific ethanol production rate ($\text{g}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$)	0.04	0.03	0.06
Specific xylose uptake rate ($\text{g}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$)	0.11	0.09	0.13

Oxygen limitation and xylose induce fermentation in *P. stipitis*



Other accomplishments:

- Identified mutations that relieve the toxic effect of xylulokinase over expression
- Identified additional genes that relieve the toxic effect when over expressed in engineered cells
- In cooperation with DOE JGI, sequenced the 15 Mbp *P. stipitis* genome
- Developed an efficient transformation and marker recovery system for *P. stipitis*
- Identified missing genes in *P. stipitis* that could contribute to anaerobic growth and ethanol tolerance



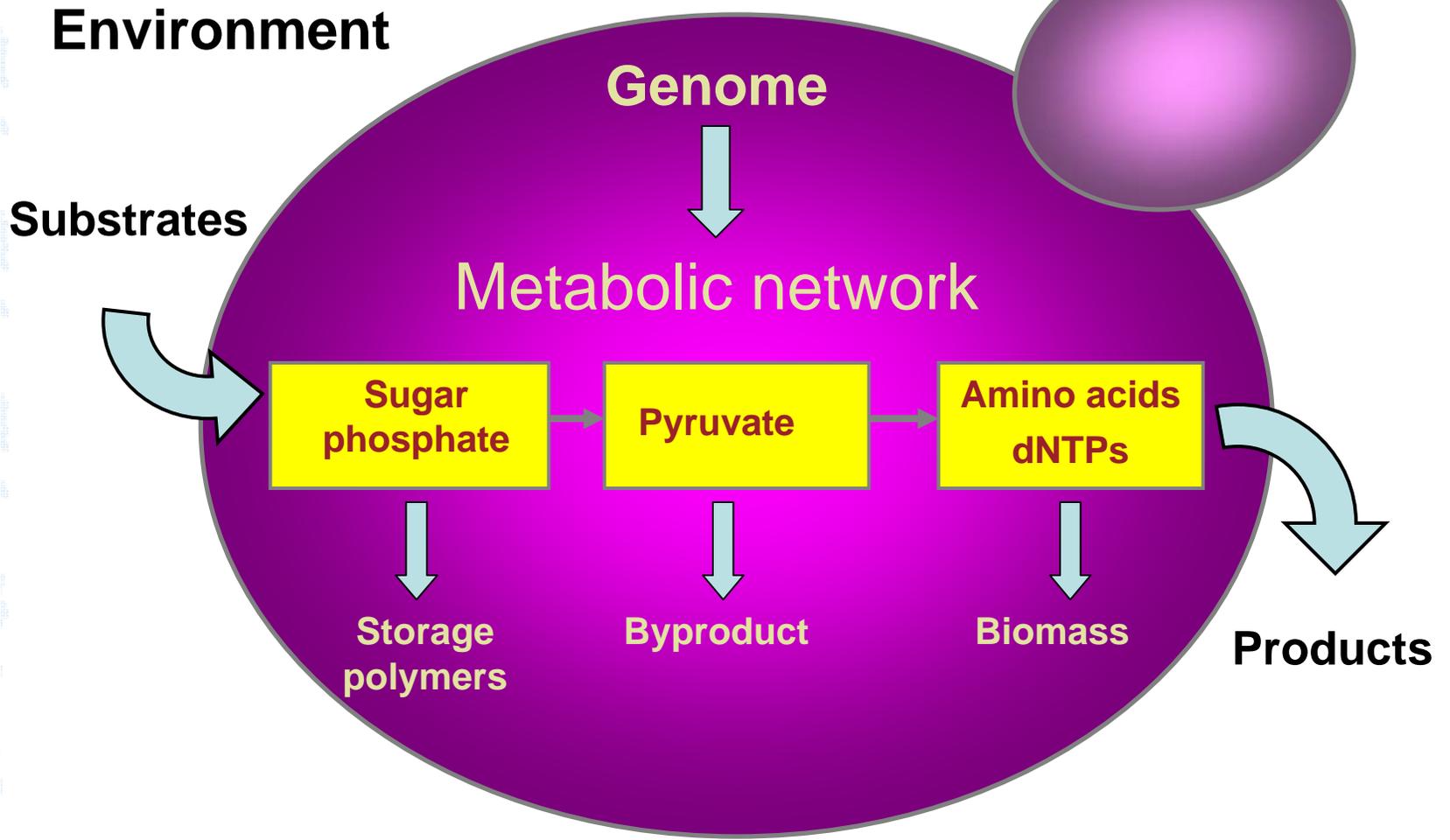
Social impact of this research

- Expand the resource base for renewable transportation fuels
- Increase farm incomes through higher value use of residues
- Increase the profitability of hardwood pulping in the U.S.
- Increase employment opportunities in rural regions

Acknowledgements

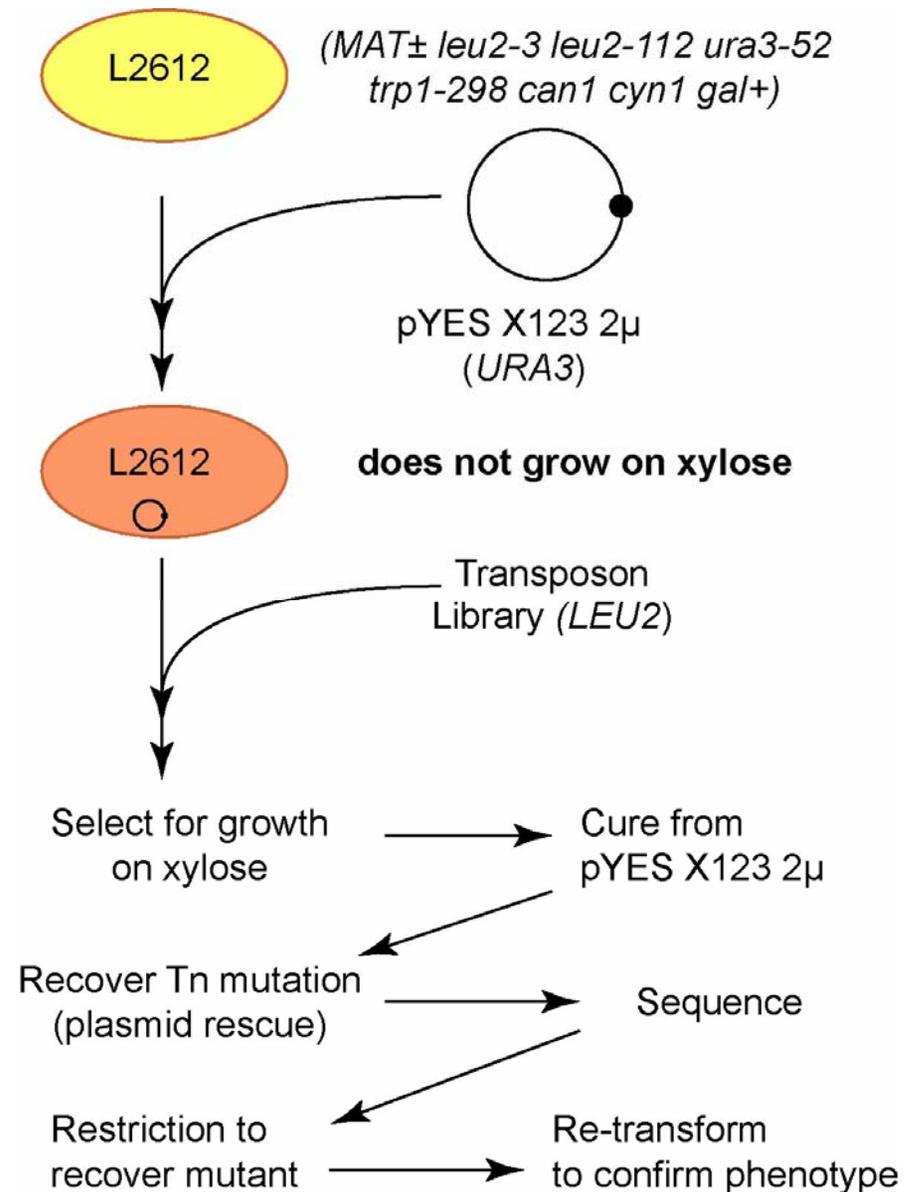
- 
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 - Karen Mansorabadi
 - Chen-Feng Lu
 - Jennifer Headman
 - Ju Yun Bae

Yeast metabolic network

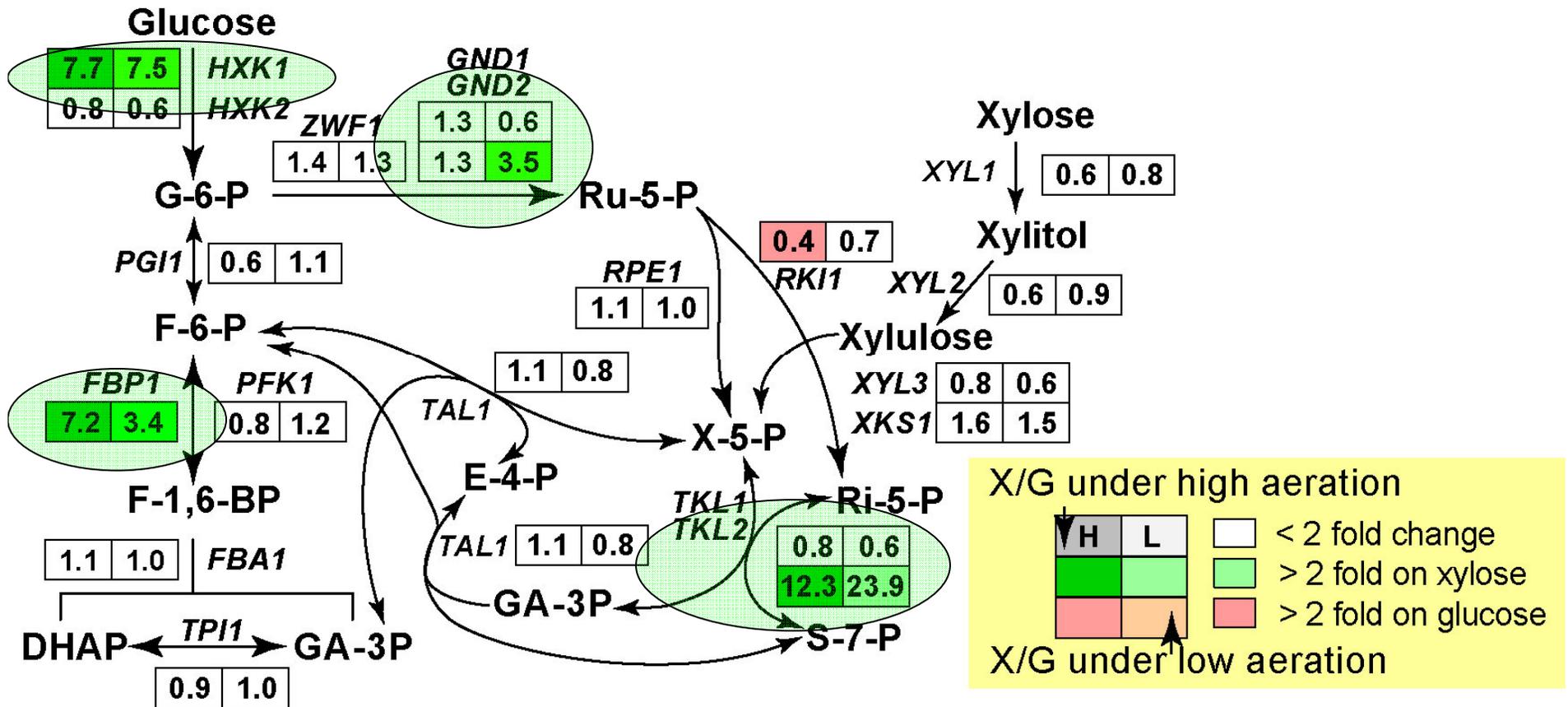


Transposon mutagenesis

- Parental strain L2612 transformed with a plasmid over expressing *XYL1,2,3*
- This genotype does not grow on xylose
- Transposon mutated library was introduced and resulting *Leu*⁺ transformants were screened for growth
- Two specific genes were identified

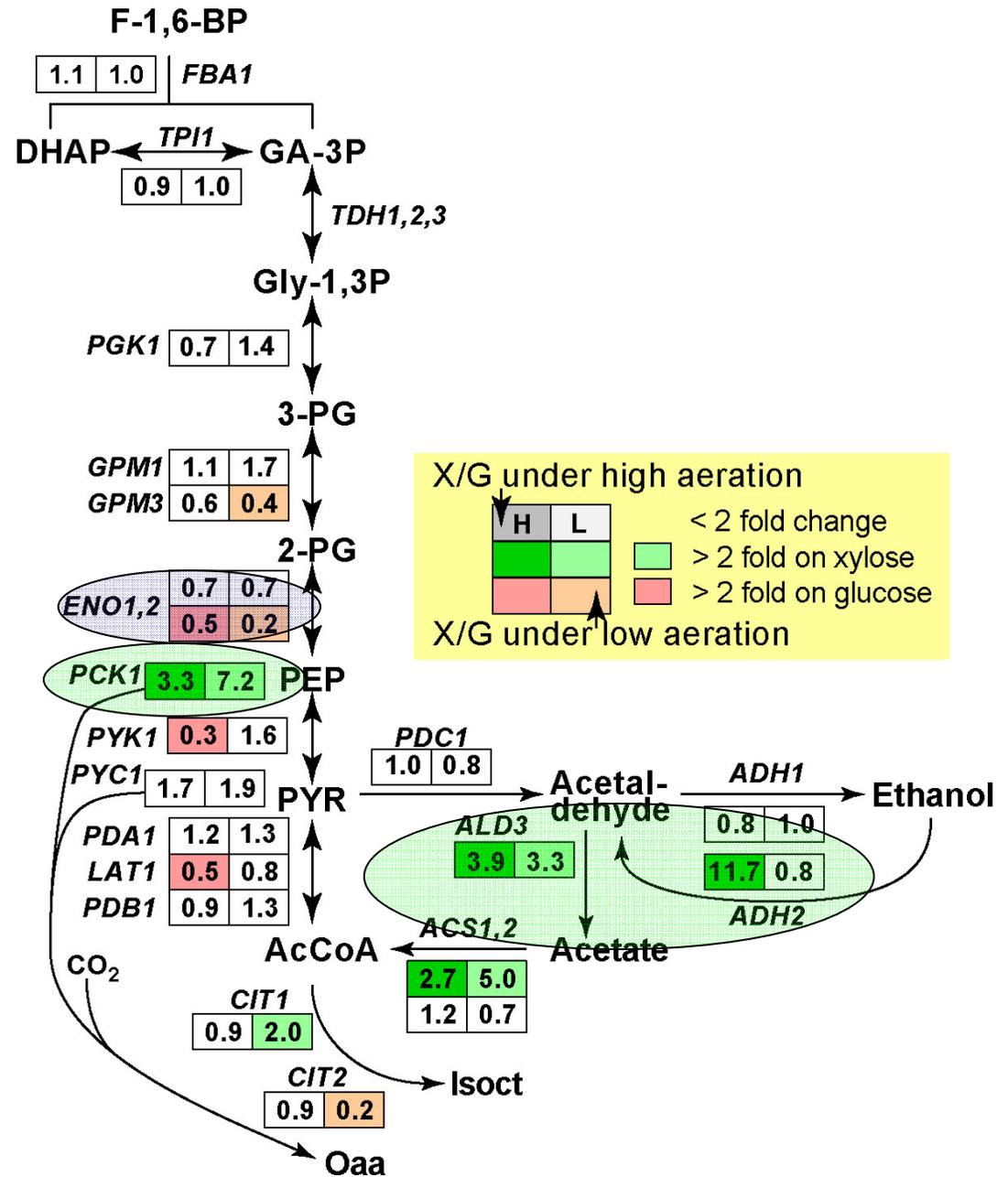


H XK1, FBP1, GND2 and TKL2 are induced on xylose

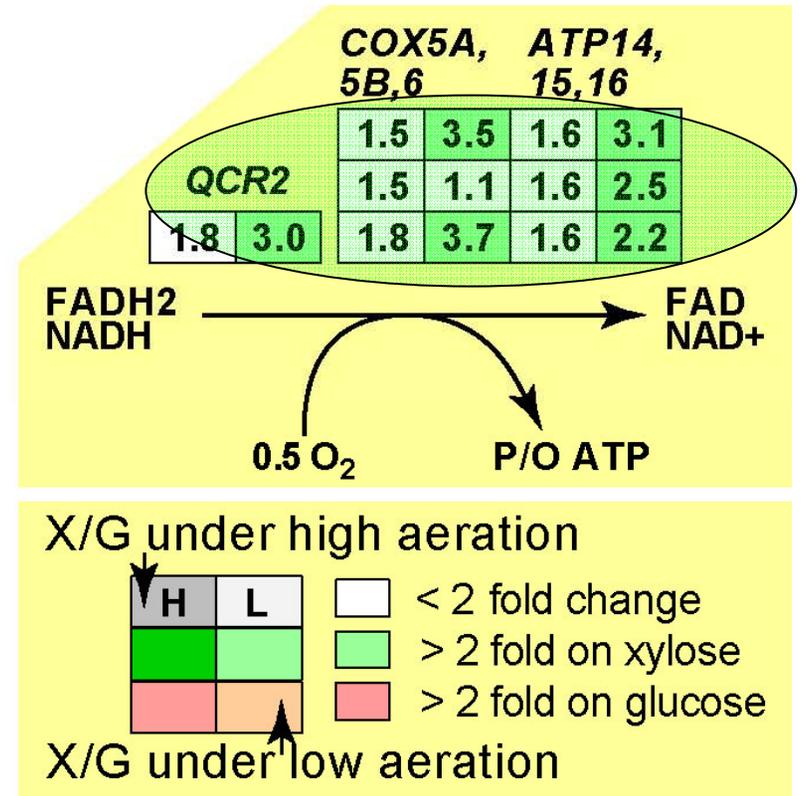
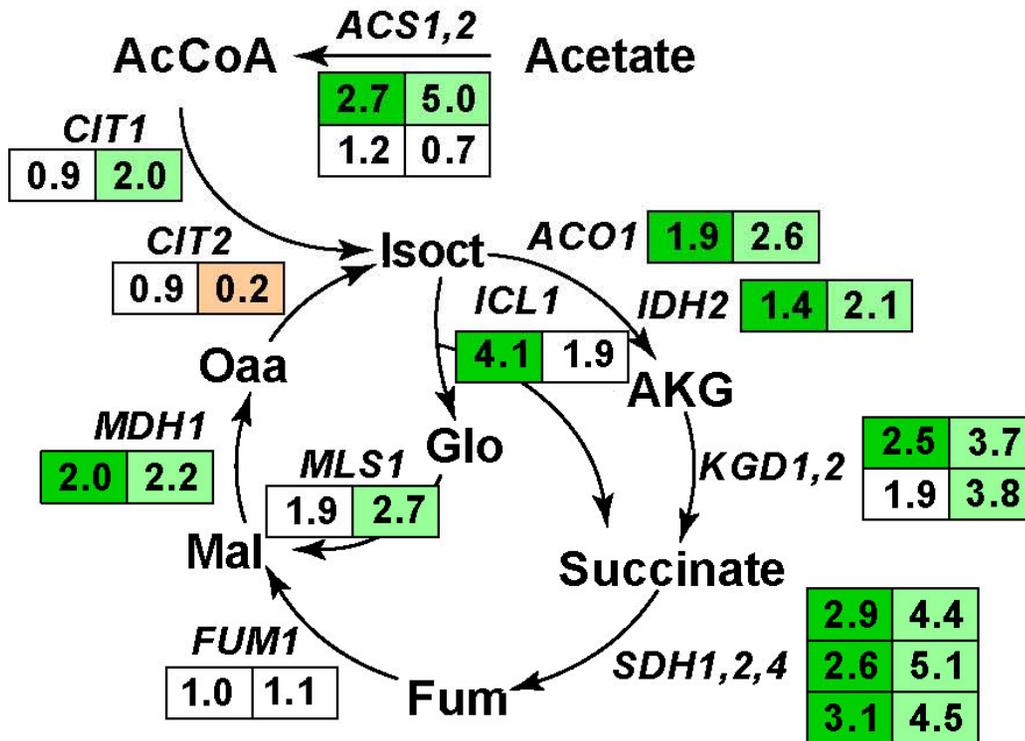


Glycolytic pathway diverges at PEP

- Most transcripts are unchanged on glucose and xylose
- PEP carboxykinase (*PCK1*) is induced on xylose
- Enolase 2 (*ENO2*) is induced on glucose
- ADH2* and *ALD3* are induced on xylose under aerobic conditions



Transcripts for TCA and respiration are induced on xylose - especially under oxygen limitation





S. cerevisiae engineered for xylose metabolism

- Requires oxygen for growth on xylose
- Induces transcripts for respiration on xylose
- Petite mutants of engineered yeast show increased fermentation
- Petite mutants do not grow on xylose
- Want to better understand limitations